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Tissue Factor Residues That Modulate Magnesium-Dependent Rate Enhancements of the Tissue Factor/Factor VIIa Complex

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Abstract

The blood coagulation cascade is initiated when the cell-surface complex of factor VIIa (FVIIa, a trypsin-like serine protease) and tissue factor (TF, an integral-membrane protein) proteolytically activates factor X (FX). Both FVIIa and FX bind to membranes via their γ-carboxyglutamate-rich domains (GLA domains). GLA domains contain seven to nine bound Ca^{2+} ions that are critical for their folding and function, and most biochemical studies of blood clotting have employed supraphysiologic Ca^{2+} concentrations to ensure saturation of these domains with bound Ca^{2+}. Recently, it has become clear that, at plasma concentrations of metal ions, Mg^{2+} actually occupies two or three of the divalent metal ion-binding sites in GLA domains, and that these bound Mg^{2+} ions are required for full function of these clotting proteins. In this study, we investigated how Mg^{2+} influences FVIIa enzymatic activity. We found that the presence of TF was required for Mg^{2+} to enhance the rate of FX activation by FVIIa, and we used alanine-scanning mutagenesis to identify TF residues important for mediating this response to Mg^{2+}. Several TF mutations, including those at residues G164, K166, and Y185, blunted the ability of Mg^{2+} to enhance the activity of the TF/FVIIa conplex. Our results suggest that these TF residues interact with the GLA domain of FX in a Mg^{2+}-dependent manner (although effects of Mg^{2+} on the FVIIa GLA domain cannot be ruled out). Notably, these TF residues are located within or immediately adjacent to the putative substrate-binding exosite of TF.

Keywords

Tissue Factor; Factor VIIa; Factor X; Factor IX; Magnesium; Calcium; Extrinsic Tenase Complex

The importance of divalent metal ions in blood coagulation reactions is well-documented, as they play key roles in both protein-membrane and protein-protein interactions.[2,3] Seven

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Supporting Information: A table listing the initial rates of FX activation supported by all TF mutants under three different divalent metal ion conditions, graphs of the effects of Mg^{2+} on rates of FIX activation by TF/FVIIa (for WT and mutant TF), a graph showing the effects of increasing the Ca^{2+} concentration on FX activation by TF/FVIIa (for WT and mutant TF), a sequence alignment of the mutated portion of TF from several mammalian species, and the K_d values for binding of FVIIa to TF (both WT and selected mutants). The Supporting Information is available free of charge on the ACS Publications website DOI: 10.1021/acs.biochem.5b00608.

blood coagulation proteins interact reversibly with anionic membranes via their GLA domains, which are rich in the post-translationally modified amino acid, γ-carboxyglutamate (Gla). GLA domains bind multiple divalent metal ions,[4] causing them to fold and form a characteristic ω-loop structure, exposing hydrophobic residues that are believed to facilitate penetration of the membrane bilayer.[5]

The vast majority of biochemical studies of blood clotting proteins have employed supraphysiologic concentrations of Ca^{2+}, which can occupy seven to nine metal ion-binding sites in the GLA domains of factors VII (FVII), IX (FIX), and X (FX).[4, 6-8] Ca^{2+} is the most prevalent divalent metal ion in plasma, with a free concentration of ∼1.25 mM.[9] However, biochemical studies of blood clotting proteins using 1.25 mM Ca^{2+} result in submaximal enzymatic activity and membrane binding, apparently because of incomplete saturation of the metal ion-binding sites in the GLA domains of these proteins. For this reason, supraphysiologic Ca^{2+} concentrations (typically, 2.5-5 mM Ca^{2+}) are typically employed in studies of blood clotting reactions. Recent studies, however, have shown that using the plasma concentrations of free Ca^{2+} (1.25 mM) together with the plasma concentration of free Mg^{2+} (∼0.6 mM) results in clotting factor activities that are at least as high as those observed in the presence of supraphysiologic Ca^{2+} concentrations.[10-14] These studies therefore indicate that Mg^{2+} plays important, albeit somewhat overlooked, roles in blood clotting reactions.

In the study presented here, we examined how Mg^{2+} modulates the initiation phase of the blood clotting cascade. Blood clotting is triggered when the integral membrane protein, tissue factor (TF), binds FVIIa to form the TF/FVIIa complex. This complex activates FIX and FX by limited proteolysis. The mechanism(s) by which Mg^{2+} modulates the activity of clotting proteases is unclear, but recent crystal structures of FVIIa indicate that Mg^{2+} occupies two or three of the metal ion-binding sites in its GLA domain at physiologic concentrations of Ca^{2+} and Mg^{2+},[13] with structural implications.[1] Persson *et al.*[14] reported that removal of the FX GLA domain eliminated the ability of Mg^{2+} to increase the rate of FX activation by TF/FVIIa, suggesting that incorporation of Mg^{2+} into the GLA domain of FX may be the basis by which Mg^{2+} enhances FX activation.

We utilized site-directed mutagenesis of TF to investigate how Mg^{2+} modulates the activity of the TF/FVIIa complex (TF/FVIIa). We confirm that TF is a key mediator of the Mg^{2+} response, despite its lack of direct interaction with either Ca^{2+} or Mg^{2+}. We now identify individual TF residues within or near its putative substrate-binding exosite that are necessary for Mg^{2+} to enhance of the rate of FX activation by TF/FVIIa. Furthermore, we demonstrate that physiologic concentrations of Ca^{2+} and Mg^{2+} support TF/FVIIa enzymatic activities higher than those in the presence of Ca^{2+} alone, an effect that was more pronounced when more physiologic lipid compositions were utilized. Our findings support the idea that TF is an integral component of macromolecular substrate recognition by the TF/FVIIa complex, and that Mg^{2+} contributes to TF/FVIIa function.

Experimental Procedures

Materials

Materials were from the following sources: phospholipids 1-palmitoyl-2-oleoylphosphatidylcholine (PC) and 1-palmitoyl-2-oleoylphosphatidyl-L-serine (PS), from Avanti Polar Lipids (Alabaster, AL), Bio-Beads SM-2 absorbent from Bio-Rad, human FVIIa from American Diagnostica (now Sekisui Diagnostics, Lexington, MA), human FX, FXa, FIX, and FIXa from Haematologic Technologies (Essex Junction, VT), Spectrozyme Xa from Bachem (Bubendorf, Switzerland), Pefachrome FIXa from Enzyme Research Laboratories (South Bend, IN), and Chromozym t-PA from Roche Diagnostics (Mannheim, Germany).

Production and Relipidation of TF

Recombinant human membrane-anchored TF (memTF, residues 3-244)[15] and soluble TF (sTF, residues 3-219)[16] were expressed in *Escherichia coli* and purified as previously described. TF mutants were prepared using the Q5 site-directed mutagenesis kit from New England Biolabs (Ipswich, MA). TF liposomes were prepared by incorporating memTF into phospholipid vesicles of varying composition as described previously.[17]

FVIIa Amidolytic Activity and Quantifying TF/FVIIa Binding

Initial rates of hydrolysis of the Chromozym-tPA substrate (FVIIa amidolytic activity) were measured essentially as previously described,[18] in buffer containing either 1.25 mM $CaCl_2$, 1.85 mM $CaCl_2$, or 1.25 mM $CaCl_2$ and 0.6 mM $MgCl_2$. The affinity of FVIIa for TF was measured as described previously,[19] using 5 nM FVIIa, 0-20 nM memTF, 1 mM Chromozym-tPA, and 0.1% Triton X-100 in HBSA [20 mM HEPES (pH 7.4), 100 mM NaCl, 0.02% sodium azide, and 0.1% bovine serum albumin], either with 1.85 mM $CaCl_2$ or with 1.25 mM $CaCl_2$ and 0.6 mM $MgCl_2$.

Rates of Activation of FX and FIX

Initial rates of FX activation by TF/FVIIa using memTF, either in solution or relipidated into phospholipid vesicles, were quantified using a continuous FX activation assay as described previously,[19,20] with slight modifications. Briefly, memTF and FVIIa were incubated essentially as described, but in buffers whose divalent metal ion concentrations were either 1.25 mM Ca^{2+}, 1.85 mM Ca^{2+}, or 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+}. Reactions were initiated by addition of 100 nM FX and 1 mM Spectrozyme Xa. Reactions without membranes included 0.1% Triton X-100 and typically employed 10 nM FVIIa and 500 nM memTF. Reactions with membranes typically employed 30-100 pM FVIIa and excess relipidated memTF (>1 nM memTF, with 25 μM total phospholipid).

Initial rates of FIX activation were quantified in a two-stage assay as described previously,[21] with some variations. Briefly, FVIIa and memTF were incubated at 37°C for 5 min in buffer containing 0.06% Triton X-100 together with either 1.25 mM Ca^{2+} or 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+}. FIX was then added and incubated at 37°C, yielding typical final concentrations of 20-40 nM FVIIa, 500 nM TF, and 1 μM FIX. Timed 10 μL aliquots were removed and quenched on ice using 10 μL of 20 mM EDTA in 10×HBSA. To quantify the FIXa

generated, final concentrations of 40% ethylene glycol and 1 mM Pefachrome FIXa were added to quenched samples (total volume, 100 μL), and the rate of change in A_{405} was measured at 35°C.

Solution Nuclear Magnetic Resonance (NMR) Spectroscopy

Purified sTF was concentrated using Amicon Ultra-15 centrifugal filters (EMD Millipore, Billerica, MA) with an M_r cutoff of 10 kDa and then dialyzed into 50 mM NaCl and 50 mM sodium phosphate (pH 6.5). Solution NMR samples were prepared with 100 μM sTF, 1 mM DSS (4,4,-dimethyl-4-silapentane-1-sulfonic acid), and 10% D_2O along with 1.25 mM Ca^{2+}, 0.5 mM Mg^{2+}, or no divalent cations. ^{15}N-^{1}H two-dimensional (2D) HSQC correlation spectra were acquired on a Varian/Agilent VNMRS 17.6 T (750 MHz ^{1}H frequency) spectrometer for 16.6 h each at ∼30°C. The measured pH values were 6.74, 6.78, and 6.79 for the samples with 1.25 mM Ca^{2+}, 0.5 mM Mg^{2+}, and no divalent cations, respectively. Spectra were processed using NMRPipe[22] and chemical shifts were analyzed in SPARKY.[23]

Results

Components of the TF/FVIIa Complex Required for Mg^{2+}-Dependent Rate Enhancements

Although Mg^{2+} has been shown to modulate the enzymatic activity of FVIIa toward its cognate substrates, FIX[11] and FX,[13,24] and removal of the FX GLA domain abrogates this effect,[14] a thorough understanding of how each component of the TF/FVIIa/membrane complex responds to Mg^{2+} has not yet been achieved. We therefore varied the constituents of this complex and examined the ability of Mg^{2+} to modulate the rate of FX activation by FVIIa. Figure 1 shows the relative rates of FX activation by various components of the TF/FVIIa/membrane complex in the presence of physiologic concentrations of Ca^{2+} and Mg^{2+} (1.25 and 0.6 mM, respectively), normalized to the rates observed with physiologic (1.25 mM) Ca^{2+} alone. For FVIIa in solution (no membranes or TF), the presence of Mg^{2+} and Ca^{2+} actually decreased the rate of FX activation by 35% relative to that with Ca^{2+} alone. Mg^{2+} did not significantly influence the rate of FX activation by FVIIa in the presence of 30% PS/70% PC liposomes [but in the absence of TF (second bar in Figure 1)]. In the presence of sTF but without membranes, Mg^{2+} enhanced the rate of FX activation by FVIIa by approximately 4-fold, consistent with previous findings.[13,14] In the presence of sTF and 30% PS/70% PC liposomes, Mg^{2+} still enhanced the rate of FX activation by FVIIa, but to a lesser extent than in the absence of membranes. Mg^{2+} also enhanced FX activation by FVIIa bound to relipidated TF, with the magnitude of the enhancement dependent on the lipid composition. Thus, Mg^{2+} enhanced the rate of FX activation in the presence of TF liposomes containing either 100% PC or 5% PS and 95% PC but did not significantly enhance the rate of FX activation when TF-liposomes contained very high levels of PS (i.e., 30% PS and 70% PC).

TF Mutations That Diminish the Ability of Mg^{2+} To Enhance FX Activation by TF/FVIIa in Solution

Previous studies identified a putative substrate-binding region (exosite) in TF, consisting of a patch of surface-exposed residues in the C-terminal fibronectin type III domain of this protein that are critical for recognition of macromolecular substrates (FVII, FIX, and FX) by

the TF/FVIIa complex. In particular, mutating TF residue S163, K166, or Y185 to alanine substantially decreased the rates of FX activation by TF/FVIIa, while having little or no effect on the affinity of FVIIa for TF or on FVIIa amidolytic activity.[21] We previously showed that if the GLA domain of FX is removed, mutations in the TF exosite no longer influence the rate of FX activation, suggesting that the FX GLA domain interacts with the TF exosite.[25] Furthermore, Persson *et al.*[14] showed that removing the FX GLA domain abrogated the ability of Mg^{2+} to enhance FX activation by TF/FVIIa. We therefore hypothesized that Mg^{2+} enhances the rate of FX action by TF/FVIIa largely by enhancing the interaction between FX and the substrate-binding exosite region of TF. To test this idea, we mutated 17 amino acids in memTF individually to alanine, chosen by their location within or adjacent to the putative substrate-binding exosite.[21] We expressed and purified these memTF mutants and then assessed the ability of Mg^{2+} to enhance the rate of FX activation by the resulting TF/FVIIa complexes. In particular, we compared three divalent metal ion conditions: 1.25 mM Ca^{2+} alone, 1.85 mM Ca^{2+} alone, and 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+}. When we measured the rates of FX activation by memTF/FVIIa complexes in solution (i.e., with Triton X-100 and no membranes) in the presence of 1.85 mM Ca^{2+}, we found that 13 of the 17 single amino acid mutations decreased the initial rate of FX activation by at least 50% relative to that of wild-type (WT) memTF, and that six of the mutations (Y157A, K159A, S163A, G164A, K166A, and Y185A) decreased the rate by at least 90% (Figure 2A). These results are in good agreement with those previously determined by Kirchhofer *et al,*[21] which were obtained in the presence of phospholipid membrane surfaces. The absolute rates of FX activation obtained under each divalent metal ion condition that we tested are given in Table S1 of Supporting Information.

To determine how these TF mutations affected the ability of Mg^{2+} to enhance the enzymatic activity of the memTF/FVIIa complex (memTF/FVIIa), we quantified initial rates of FX activation in the presence of 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+} and normalized those data to the rates using 1.85 mM Ca^{2+} alone (Figure 2B) or 1.25 mM Ca^{2+} alone (Figure 2C). The purpose of the former condition is to compare the combination of Ca^{2+} and Mg^{2+} to an identical concentration of Ca^{2+} alone, such that the total divalent metal ion concentration is held constant at 1.85 mM.

Similar to the effect of Mg^{2+} we observed with sTF in solution, the combination of 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+} enhanced the rate of FX activation by FVIIa bound to WT memTF in detergent solution by 4.4-fold compared to that with 1.85 mM Ca^{2+} alone (Figure 2B) and 6.0-fold compared to that with 1.25 mM Ca^{2+} alone (Figure 2C). The TF exosite mutants fell into two main categories with regard to their sensitivity to Mg^{2+}. The first category included mutations that were highly deleterious for FX activation but that nevertheless showed essentially the same ability of Mg^{2+} to enhance the rate of FX activation observed with WT memTF/FVIIa complexes. Thus, TF mutants W158A, S160A, S162A, E174A, L176A, and R200A exhibited absolute rates of FX activation that were <75% of that of WT memTF (Figure 2A) yet showed essentially the same response to the combination of Ca^{2+} and Mg^{2+} versus Ca^{2+} alone that was observed for WT memTF (Figure 2B, C). The second category included mutants Y157A, S163A, G164A, K159A, K165A, K166A and Y185A that exhibited both substantially decreased absolute rates of FX activation (Figure 2A) and substantially blunted ability of Mg^{2+} to enhance their rates of FX activation (Figures 2B, C).

On the other hand, no TF mutants that retained WT absolute rates of FX activation yet were deficient in their response to Mg^{2+} were identified. A similar pattern of results was obtained when we examined the ability of Mg^{2+} to enhance the rate of FIX activation by this collection of memTF mutants (Figure S1 of Supporting Information).

To better understand our results, we also investigated the effect of simply increasing the Ca^{2+} concentration from 1.25 to 1.85 mM on the rate of FX activation by memTF/FVIIa complexes in solution (Figure S2 of Supporting Information). With WT TF, increasing the Ca^{2+} concentration resulted in an approximately 1.3-fold increase in the rate of FX activation. Within experimental error, most of the TF mutants supported similar increases in the rate of FX activation with an increase in the Ca^{2+} concentration. Two TF mutants (K159A and K165A) were significantly different, exhibiting approximately 30% *slower* rates of FX activation at 1.85 mM Ca^{2+} than at 1.25 mM Ca^{2+} (Figure S2 of the Supporting Information).

TF Mutations That Diminish the Ability of Mg^{2+} To Enhance FX Activation by TF/FVIIa on Membranes

Our studies thus far have examined the ability of Mg^{2+} to increase the rate of FX by TF/FVIIa complexes in solution. We next examined the effects of 11 selected TF mutants in experiments in which memTF was incorporated into liposomes of varying the phospholipid composition (Figure 3). As was also seen in Figure 1, the ability of the combination of 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+} to enhance the rate of FX activation relative to that with 1.85 mM Ca^{2+} alone using WT TF was a function of the PS content of the TF liposomes. Thus, as the PS content increased, the ability of Mg^{2+} to enhance the rate of FX activation was decreased, reaching an essentially negligible level of enhancement by Mg^{2+} at 15% PS. For the sake of clarity, we grouped the TF mutants in three panels of Figure 3 according to their ability to support Mg^{2+}-dependent enhancement of the rate of FX activation in solution. Thus, those memTF mutants that supported essentially WT responses to Mg^{2+} in solution (W158A, S162A, E174A, and D178A) also exhibited increased rates of FX activation in the presence of Mg^{2+} when employed in liposomes (Figure 3A). Although increasing the PS content of the TF liposomes tended to blunt the Mg^{2+} response in these mutants, the effect of increasing the PS content on the Mg^{2+} response was generally less extensive than that observed with WT TF. This was most particularly true of the S162A mutant.

On the other hand, the memTF mutants that supported intermediate responses to Mg^{2+} in solution (Y157A, K159A, S163A, and K165A) exhibited essentially no response to Mg^{2+} in liposomes (Figure 3B). Finally, the memTF mutants that exhibited the most deficient responses to Mg^{2+} in solution (G164A, K166A and Y185A) actually exhibited, in liposomes, *lower* rates of FX activation in the presence of Mg^{2+} and Ca^{2+} compared to the rates with Ca^{2+} alone (Figure 3C). Furthermore, the blunted responses to Mg^{2+} by the TF mutants in panels B and C of Figure 3 were observed irrespective of the PS content of the memTF liposomes.

Solution NMR Spectroscopy of sTF in the Presence of Divalent Cations

To investigate whether TF interacts directly with the divalent cations Ca^{2+} and Mg^{2+}, we utilized solution NMR spectroscopy to compare ^{15}N-^{1}H 2D heteronuclear single-quantum coherence (HSQC) correlation spectra in the presence and absence of these ions. As shown in Figure 4, addition of a physiologic divalent cation (either 1.25 mM Ca^{2+} or 0.5 mM Mg^{2+}) to 100 μM sTF samples resulted in no significant shifts in peak positions in ^{15}N-^{1}H 2D correlation spectra. These results are consistent with the idea that neither Ca^{2+} nor Mg^{2+} interacts directly with sTF in a specific manner, which in turn is consistent with available x-ray crystal structures in which neither Ca^{2+} nor Mg^{2+} has been observed bound to sTF.[8,13]

Discussion

Divalent cations such as Ca^{2+} and Mg^{2+} are critical components of blood coagulation, supporting both protein-protein and protein-membrane interactions. While the contributions of Ca^{2+} to blood coagulation reactions have been extensively studied, the roles of Mg^{2+} are less well understood. In this study, we confirmed that TF is required for Mg^{2+} to enhance the rate of activation of FIX or FX by FVIIa,[14] and we showed that specific residues in the putative substrate-binding region of TF contribute to this Mg^{2+}-dependent rate enhancement. Although TF is an allosteric regulator of the general catalytic activity of FVIIa, this function is independent of the presence of Mg^{2+} as long as Ca^{2+} is available, because adding Mg^{2+} has no significant effect on FVIIa amidolytic activity[14] and does not enhance the affinity of FVIIa for TF (Table S2 of the Supporting Information). Thus, enhancement of TF/FVIIa catalytic activity by Mg^{2+} is restricted to macromolecular substrates (FIX and FX). Importantly, a previous study showed that removing the FX GLA domain eliminated the ability of Mg^{2+} to enhance the rate of FX activation by TF/FVIIa,[14] and our lab previously demonstrated that the GLA domain of FX must be intact for mutations in the TF exosite to have any effect on the rate of FX activation by TF/FVIIa.[25] These results support the idea that the FX GLA domain interacts directly with the TF exosite and is of critical importance for Mg^{2+} recognition. This does not preclude the involvement of FVIIa in mediating these interactions; indeed, a recent study reported that the FVIIa GLA domain must also be intact for Mg^{2+} to enhance the rate of FX activation by TF/FVIIa.[26]

Our working hypothesis to explain all these findings is that Mg^{2+} promotes the binding of FX (or FIX) to the exosite region of TF. To accomplish this, Mg^{2+} could, in principle, be contributing to the increased rates of FX activation via its binding to the GLA domains of either FVIIa or FX, or perhaps even binding to TF. (Because we observe the effect of Mg^{2+} in solution, it is not necessary to invoke association of Mg^{2+} with phospholipids, and therefore, Mg^{2+} must be promoting protein-protein interactions.) Our NMR results, together with a wealth of X-ray crystal structures, argue strongly that Mg^{2+} does not associate measurably with TF. At physiologic concentrations of divalent metal ions, Mg^{2+} is thought to occupy at least two, and possibly three or four metal ion-binding sites in the GLA domains of both FVIIa and FX.[27] Although our results do not allow us to determine whether binding of Mg^{2+} to FVIIa or FX is more important for enhancing the rate of FX activation, one can speculate that occupancy of the FX GLA domain with Mg^{2+} may increase the affinity of this domain for the TF exosite. Binding of Mg^{2+} to the FVIIa GLA domain may

also play a regulatory role, as comparison of crystal structures in the presence or absence of Mg^{2+} indicates that conformational changes occur in the FVIIa GLA domain upon Mg^{2+} occupancy.[1] Interestingly, the 159-165 loop of sTF was disordered when the sTF/FVIIa complex was crystallized with Ca^{2+} only but was ordered when the complex was crystallized in the presence of both Ca^{2+} and Mg^{2+}. How these structural changes might affect TF/FVIIa activity toward its cognate substrates is currently unclear.

For better visual interpretation, we mapped the mutations in this study onto the X-ray crystal structure of the FVIIa/sTF complex, colored according to their impact either on the absolute rate of FX activation (Figure 5A) or on the ability of Mg^{2+} to enhance the rate of FX activation (Figure 5B) by memTF/FVIIa in solution. In Figure 5C, we also graphed the effects of these mutations, plotted as the ability of Mg^{2+} to enhance the rate of FX activation (*y*-axis) versus the absolute rate of FX activation (*x*-axis), with data points colored using the same scheme as in Figure 5B. The deleterious effects of these TF exosite mutations on the absolute rate of FX activation (Figure 5A) largely confirm the findings of Kirchhofer et al.[21] Only a subset of these mutations, however, weakened the ability of Mg^{2+} to enhance the rate of FX activation, with mutations G164A, K166A, and Y185A (colored blue in Figure 5B) essentially eliminating the Mg^{2+} effect; notably, these three mutations also severely reduced the absolute rate of FX activation (colored blue in Figure 5A). TF mutations Y157A, S163A, K165A, and D180A (colored green in Figure 5B) exhibited a more moderate yet still significant reduction in the Mg^{2+} effect; of these, three (Y157A, S163A, and K165A) greatly reduced the absolute rate of FX activation (colored blue in Figure 5A), while one (D180A) produced a more moderate reduction in the absolute rate of FX activation (colored green in Figure 5A). TF mutant K159A severely reduced the absolute rate of FX activation but did not significantly diminish the Mg^{2+} effect relative to that of WT TF. The 10 remaining mutations also did not significantly weaken the ability of Mg^{2+} to enhance the rate of FX activation relative to that of WT TF, irrespective of whether they decreased the absolute rate of FX activation.

The location of TF residues that mediate Mg^{2+}-dependent rate enhancement suggests that a critical component of the TF/FVIIa interaction with Mg^{2+}-bound FX substrate involves the flexible TF loop located at residues 160-165, with the adjacent residues Y185, K166, K159, and Y157 also involved. These residues, in particular residues 164-166, are highly conserved across mammalian species (Figure S3 of Supporting Information). The importance of G164 to Mg^{2+} recognition is particularly interesting, as glycine lacks a functional side chain with which to interact with macromolecular substrates. A possible explanation is that this highly conserved glycine permits flexibility or orientations of the adjacent loop(s) that are otherwise not possible with amino acids containing bulkier side chains.

When WT memTF was incorporated into liposomes, we found that increasing the PS content of the membranes blunted the response to Mg^{2+}, confirming our previous findings.[24] We also found that increasing the PS content of TF liposomes blunted the Mg^{2+} response in these TF mutants. With several of these mutants (especially S162A), this blunting effect of PS was less extensive than that observed with WT TF. We also found that the TF mutants that were most deficient in response to Mg^{2+} when tested in solution (G164A, K166A, and Y185A) actually had *lower* rates of FX activation in TF liposomes in the presence of Mg^{2+}

and Ca^{2+} than in the presence of Ca^{2+} alone. Furthermore, the essentially negative effects of Mg^{2+} on this class of TF mutants were observed irrespective of the PS content of the memTF liposomes.

Messer et al. have shown that occupancy of the FIXa GLA domain with Mg^{2+} enhances its affinity for PS-containing membranes and have proposed that Mg^{2+} generally enhances the membrane binding of GLA domain-containing clotting proteins.[28] If this is part of the mechanism by which Mg^{2+} enhances the rate of FX activation by TF/FVIIa (i.e., by better recruiting FX to the membrane surface in the vicinity of TF/FVIIa and thereby increasing the local substrate concentration), then it follows that increasing the PS content may blunt the Mg^{2+} effect, because greatly increasing the PS content will likewise increase the affinity of FX for the membrane surface and thus overshadow the relative contribution from Mg^{2+}. However, the fact that some TF mutations selectively abolish the ability of Mg^{2+} to enhance the rate of FX activation by the TF/FVIIa/membrane complex argues in favor of protein-protein interactions (presumably, between the TF exosite and the GLA domain of FX) being also very important in mediating the ability of Mg^{2+} to enhance the proteolytic activity of TF/FVIIa toward FIX and FX.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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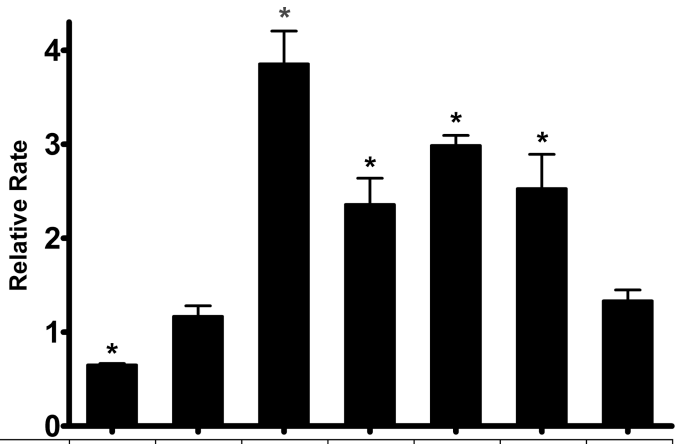
Abbreviations

FX	factor X
FIX	factor IX
FVII	factor VII
HSQC	heteronuclear single quantum coherence
memTF	tissue factor (residues 3-244) containing its membrane-anchoring helix
NMR	nuclear magnetic resonance
sTF	soluble tissue factor (residues 3-219)
TF	tissue factor



[FVIIa]	100 nM	100 pM	10 nM	15 pM	100 pM	3 pM	3 pM
TF	–	–	sTF	sTF	memTF	memTF	memTF
Liposomes (25 μM lipids)	–	30PS/ 70PC	–	30PS/ 70PC	100PC	5PS/ 95PC	30PS/ 70PC

Figure 1.

Effect of Mg^{2+} on FX activation by FVIIa in the presence or absence of TF or membranes. FX activation by FVIIa was measured in the presence of the components listed, with a dash (—) indicating the component was not present. Data are mean initial rates of FX activation in the presence of 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+} normalized to rates using 1.25 mM Ca^{2+} alone. Error bars are one standard error ($n \geq 3$). Normalized rates that are statistically significantly different from 1.0 are indicated with an asterisk (one-sample *t*-tests; $p < 0.05$).

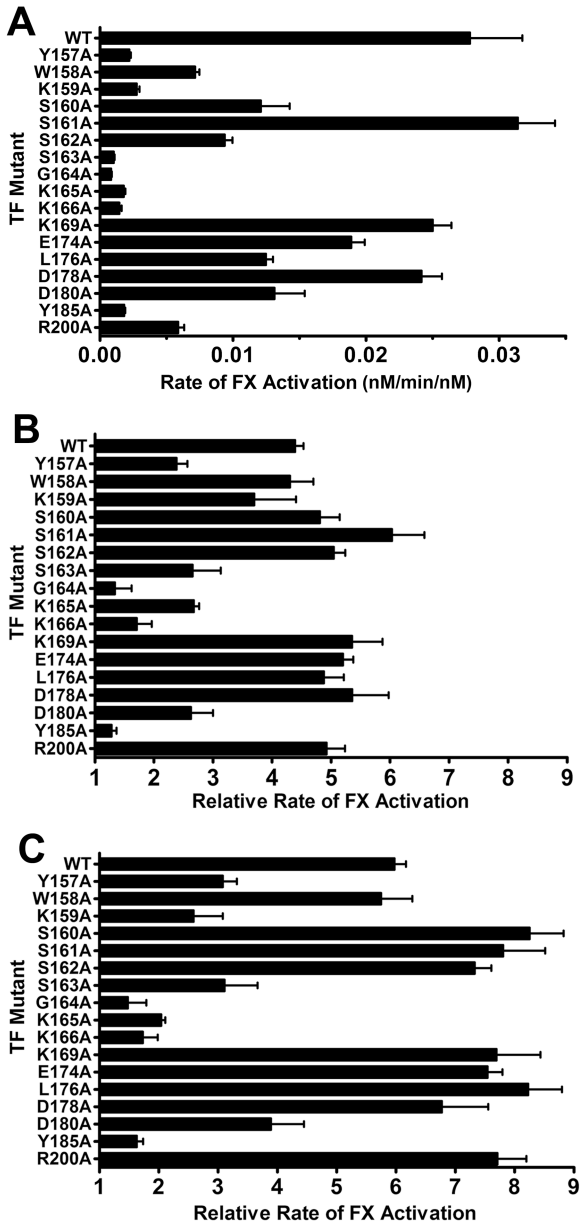


Figure 2.

Effect of TF mutations on rates of FX activation by the memTF/FVIIa complex in solution (with 0.1% Triton X-100), measured using different divalent metal ion concentrations. (A) FX activation by the memTF/FVIIa complex using 1.85 mM Ca^{2+} alone, graphed as initial rates of FX activation divided by the memTF/FVIIa complex concentration. (B) Relative rates of FX activation by memTF/FVIIa in solution using 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+}, normalized to rates using 1.85 mM Ca^{2+} alone. (C) Relative rates of FX activation by the memTF/FVIIa complex in solution using 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+}, normalized to rates using 1.25 mM Ca^{2+} alone. Data are means ± the standard error ($n \geq 3$).

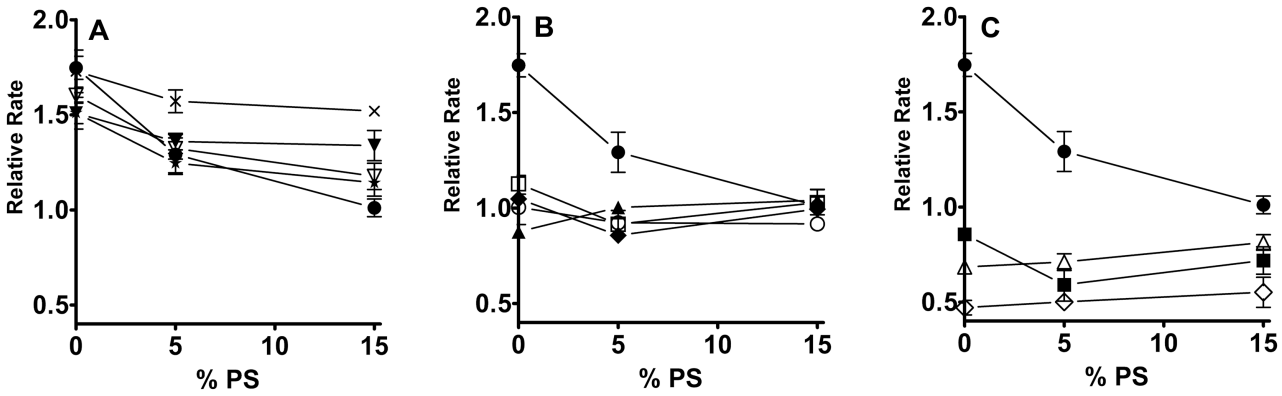


Figure 3.

Effect of 11 selected TF mutations on the ability of Mg^{2+} to enhance the rate of FX activation by TF/FVIIa/membrane complexes. WT or mutant memTF was incorporated into liposomes containing 0-15% PS, with the balance being PC. Initial rates of FX activation by the resulting memTF/FVIIa complexes were measured with 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+} and normalized to the rates observed with 1.85 mM Ca^{2+} alone. The memTF mutants are grouped in the three panels as described in the text, with the same data for WT memTF (●) plotted in each panel for comparison. (A) Normalized rates of FX activation observed with memTF mutants S162A (×), W158A (▼), E174A (▽), and D178A (★). (B) Normalized rates of FX activation observed with memTF mutants S163A (□), K165A (◆), K159A (○), and Y157A (▲). (C) Normalized rates of FX activation observed with memTF mutants Y185A (Δ), K166A (■), and G164A (◊). Data are means ± the standard error ($n \geq 3$).

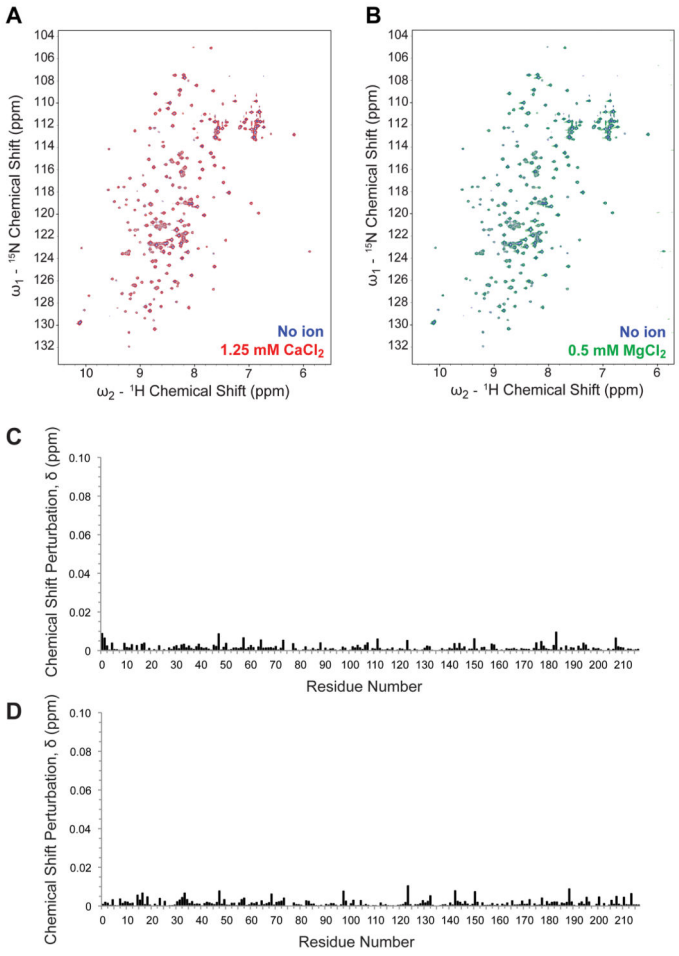


Figure 4.

Lack of a detectable effect of divalent metal ions on NMR spectra of sTF. (A) ^{15}N-^{1}H 2D HSQC correlation spectrum of 100 μM sTF in 50 mM sodium phosphate, 50 mM NaCl, 1 mM DSS, 10% D_2O and no divalent metal ions (blue) overlaid with the sTF spectrum in the same buffer with 1.25 mM Ca^{2+} (red). (B) ^{15}N-^{1}H 2D HSQC correlation spectrum of 100 μM sTF in the same buffer as in panel A, without divalent metal ions (blue) overlaid with the spectrum in the presence of 0.5 mM Mg^{2+} (green). (C) Chemical shift perturbations upon titration with 1.25 mM Ca^{2+} (from panel A) calculated as $\delta = ([0.1\delta_N]^2 + \delta_H^2)^{1/2}$), with an average chemical shift perturbation of 0.0014 ppm. (D) Chemical shift perturbations upon titration with 0.5 mM Mg^{2+} (from panel B), with an average chemical shift perturbation of 0.0015 ppm. Of the 208 expected non-proline resonances in sTF, 196 resonances were assigned.

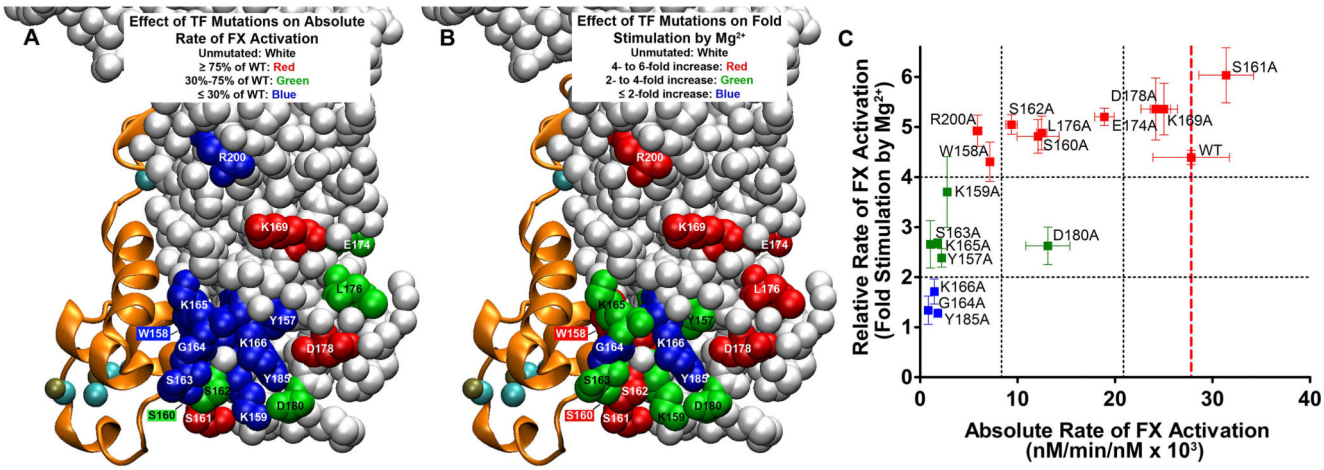


Figure 5.

Localization and properties of TF residues investigated. The structure is from Protein Data Bank entry 3TH2, in which sTF/FVIIa was crystallized in the presence of 5 mM Ca^{2+} and 2.5 mM Mg^{2+}.[1] FVIIa is depicted as orange ribbons, with bound Ca^{2+} ions colored teal and Mg^{2+} ions beige. (A) Localization of the TF residues tested in this study for their effect on the absolute rate of FX activation by memTF/FVIIa in solution. TF residues are color-coded according to their rate of FX activation in the presence of 1.85 mM Ca^{2+} alone (from Figure 2A). Unmutated TF residues are colored white. TF residues are colored red, which, when mutated, retained essentially WT activity (i.e., ≥ 75% of the WT rate of FX activation). Residues with a moderate effect on the FX activation rate are colored green (30-75% of the WT rate); residues with severe defects are colored blue (≤ 30% of the WT rate). Residues W158 and S160, obstructed in this view, are colored blue and green, respectively. (B) Localization of TF residues tested in this study as being important for the ability of Mg^{2+} to enhance the rate of FX activation by memTF/FVIIa in solution. TF residues are color-coded according to their rate of FX activation in the presence of Ca^{2+} and Mg^{2+} normalized to the rate at 1.85 mM Ca^{2+} alone (from Figure 2B). Unmutated TF residues are colored white. TF residues are colored red, which, when mutated, retained essentially WT responses to Mg^{2+} (i.e., 4-6-fold enhancement of the FX activation rate). Residues with blunted responses to Mg^{2+} are colored green (2-4-fold enhancement by Mg^{2+}) or blue (1-2-fold enhancement by Mg^{2+}). Residues W158 and S160, obstructed in this view are colored red. (C) Relative rate of FX activation (rate with 1.25 mM Ca^{2+} and 0.6 mM Mg^{2+}, divided by the rate with 1.85 mM Ca^{2+}) vs the absolute rate of FX activation, in both cases by memTF/FVIIa in solution (i.e., data replotted from panels A and B of Figure 2, respectively). Residues are color-coded as in panel B. Vertical black dotted lines correspond to the color-coding cutoff values from panel A; horizontal black dotted lines correspond to the color-coding cutoff values from panel B. A vertical dashed red line marks the location of WT TF.